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Oxidative stress and dysregulation of superoxide dismutase and NADPH oxidase in renal insufficiency

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Oxidative stress and dysregulation of superoxide dismutase and NADPH oxidase in renal insufficiency.

Background. Chronic renal failure (CRF) is associated with oxidative stress, the mechanism of which remains uncertain. Superoxide is the primary oxygen free radical produced in the body, NAD(P)H oxidase is the major source of superoxide production and superoxide dismutase (SOD) is responsible for removal of superoxide. We hypothesized that CRF-induced oxidative stress may be due to increased production and/or decreased dismutation of superoxide.

Methods. Immunodetectable superoxide dismutase isoforms (Cu Zn SOD and Mn SOD), as well as, NAD(P)H oxidase (gp91 phox subunit) proteins and xanthine oxidase (XO) activity were determined in the kidney and liver of CRF (5/6 nephrectomized) and sham-operated control rats. Subgroups of animals were treated with SOD-mimetic drug, tempol and blood pressure and urinary nitric oxide metabolites (NO_x) were monitored.

Results. The CRF group showed marked down-regulations of CuZn SOD and Mn SOD and significant up-regulation of gp91 phox in the liver and kidney, which are among the metabolically most active tissues. In contrast, XO activity was depressed in both tissues. Arterial pressure and nitrotyrosine abundance were elevated while urinary NO_x excretion was depressed, pointing to increased NO inactivation by superoxide and decreased NO availability in CRF animals. Administration of SOD-mimetic agent, tempol, for one week, ameliorated hypertension, reduced nitrotyrosine abundance and increased urinary NO_x excretion in the CRF animals.

Conclusions. CRF is associated with depressed SOD and elevated NAD(P)H oxidase expression, which can contribute to oxidative stress by increasing superoxide. This is evidenced by favorable response to administration of SOD-mimetic drug, tempol, and increased nitrotyrosine that is the footprint of NO interaction with superoxide.

Key words: renal failure, hypertension, nitric oxide, uremic toxins, oxygen-free radicals, anemia, cardiovascular disease, lipid peroxidation, advanced glycosylation end products, xanthine oxidase.

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Oxidative reactions that fuel various biochemical, biophysical and mechanical functions of the aerobic organisms are coupled with continuous generation of highly reactive and potentially cytotoxic reactive oxygen species (ROS). Under normal conditions, the ROS produced in the course of metabolism are contained by the natural antioxidant system that protects the functional and structural molecules against ROS-mediated modifications, thereby preventing cytotoxicity and tissue damage. The natural antioxidant system consists of a series of antioxidant enzymes as well as numerous endogenous and dietary antioxidant compounds that are capable of reacting with and inactivating ROS.

Under a variety of abnormal conditions, the rate of ROS production may exceed the natural antioxidant capacity leading to oxidative stress in which uncontained ROS can attack the functional or structural molecules, and thereby produce tissue injury and dysfunction. Oxidative stress can occur as a result of either excess ROS production, or impaired antioxidant system, or a combination thereof. The primary ROS produced in the course of oxygen metabolism is superoxide, which is a highly reactive, cytotoxic ROS. Superoxide is dismutated to a far less reactive product, hydrogen peroxide (H_2O_2), by a family of metalloenzymes known as superoxide dismutase (SOD). Thus, SOD is the front line of defense against ROS-mediated injury. Three isoforms of SOD have been identified thus far. These include the copper-zinc SOD (Cu Zn SOD), which is present in the cytoplasm, manganese SOD (Mn SOD), which is found in the mitochondria, and extracellular SOD (ECSOD), which exists in the interstitial fluid, plasma, lymph and synovial fluid [1].

Oxidative stress has emerged as a major feature of chronic renal failure (CRF). This conclusion is based on the following observations. First, plasma concentration of lipid peroxidation products is markedly elevated, denoting enhanced ROS interaction with the lipid molecules in CRF [2–5]. Second, CRF results in accumulation

of advanced glycated end products (AGE) and advanced lipoxidation end products (ALE) pointing to modification of protein molecules by ROS-mediated generation of reactive carbonyl compounds and lipoperoxides, respectively [6]. Third, recent studies from our laboratories have demonstrated enhanced ROS-mediated nitric oxide inactivation, protein nitration, functional NO deficiency and hypertension in CRF [7, 8].

The precise mechanism(s) of CRF-induced oxidative stress has not been elucidated. However, earlier studies carried out to discern the cause of shortened erythrocyte life span demonstrated a significant reduction of SOD activity and antioxidant capacity in erythrocytes of CRF patients [9–12]. It is uncertain as to whether the reported reduction of erythrocyte SOD activity is due to inhibition of SOD by the uremic milieu or an actual reduction of SOD protein. Likewise, it is unclear as to whether the reported reduction of SOD activity in CRF is confined to the uremic erythrocyte or is a systemic phenomenon. If present, systemic SOD deficiency can account, in part, for the CRF-induced oxidative stress.

Nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidases are the major source of superoxide production in phagocytes and vascular tissue [13]. Thus, up-regulation of NADPH oxidase abundance or activity can lead to oxidative stress. To our knowledge, the effect of renal insufficiency on tissue NAD(P)H oxidase has not been investigated.

We hypothesized that CRF may lead to down-regulation of SOD and up-regulation of NAD(P)H oxidase expression in major organs. We further considered that if this hypothesis is true, administration of a cell-permeable SOD-mimetic agent should ameliorate oxidative stress, reduce ROS-mediated nitric oxide inactivation and improve the associated hypertension. The present study was undertaken to test this hypothesis.

METHODS

Study groups

Male Sprague Dawley rats weighing 230 to 250 g (Harlan Sprague Dawley Inc., Indianapolis, IN, USA) were used in this study. Animals were housed in a climate-controlled space with 12-hour day and night cycles and were provided free access to regular rat chow and water. The animals were randomly assigned to the CRF and sham-operated control groups. The CRF group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of left kidney, followed by right nephrectomy four days later. The control group underwent sham operation. The procedures were carried out under general anesthesia (pentobarbital 50 mg/kg IP) using strict hemostasis and aseptic techniques. The nephrectomy procedures were accomplished via dorsal incisions as described in our earlier studies [14]. Animals were monitored for six weeks after nephrectomy and sham operation. In an attempt to discern the possible elevation of superoxide activity, subgroups of the CRF and control animals were treated with the SOD-mimetic drug, tempol (Sigma Chemical Co., St. Louis, MO, USA) added to the drinking water at 1 mmol/L for one week beginning on week 5 post-nephrectomy.

At the conclusion of the six-week observation period, animals were placed in individual metabolic cages for a timed urine collection. The animals were anesthetized (pentobarbital 50 mg/kg, IP) and exsanguinated by cardiac puncture. Remnant kidney and liver were immediately harvested, cleaned and snap-frozen in liquid nitrogen. Plasma and frozen tissues were then stored at −70°C until processed. The experimental protocol employed in the study was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Measurements of SOD and NAD(P)H oxidase proteins

Homogenates (25% wt/vol) of kidney, heart (left ventricle), thoracic aorta and brain were prepared in 10 mmol/L HEPES buffer, pH 7.4, containing 320 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 10 μg/mL leupeptin, 2 μg/mL aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) at 0 to 4°C with a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 9000 × *g* for 10 minutes at 4°C to remove nuclear fragments and tissue debris without precipitating membrane fragments. A portion of the supernatant was used for the determination of total protein concentration by using a Bio-Rad kit (Hercules, CA, USA).

Total cellular protein (Cu Zn SOD, 1 μg; Mn SOD and NADPH oxidase, 20 μg each) was electrophoresed in 4 to 20% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels (Novex, San Diego, CA, USA). Proteins were transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA, USA), blocked in 5% dry milk in T-TBS (0.02 mol/L Tris/0.15 mol/L NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for three hours, washed three times with T-TBS and incubated with the primary antibodies (both the Cu Zn SOD and Mn SOD, 1:1000; NADPH oxidase, 1:2000) for three hours at room temperature. Cu Zn SOD and Mn SOD antibodies were purchased from Calbiochem Inc. (San Diego, CA, USA). For NADPH oxidase, we used the monoclonal antibody against the gp91 phox subunit (Transduction Labs, Lexington, KY, USA). After washing five times with T-TBS, the blots were incubated with secondary antibodies (anti-sheep for both the Cu Zn and Mn SOD, 1:2000 and anti-mouse for NADPH oxidase, 1:2000) conjugated with horseradish peroxidase at room temperature for two hours. After washing five

times with T-TBS, the membrane was developed using enhanced chemiluminescent (ECL) reagent (Amersham Life Science Inc., Arlington Heights, IL, USA) and subjected to autoluminography for one to five minutes. The autoluminographs were scanned with a laser densitometer (Model PD 1211; Molecular Dynamics, Sunnyvale, CA, USA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain, to verify the uniformity of protein load and transfer efficiency across the test samples.

Measurement of arterial pressure

Arterial pressure was determined by tail plethysmography (Harvard Apparatus, Natick, MA, USA) as described in our earlier studies [5].

Measurement of urinary NO metabolites (NO_x)

Urinary concentration of NO_x (total NO_2 and NO_3) was determined by means of the Sievers model 270B nitric oxide analyzer (Sievers Instruments, Boulder, CO, USA) as described in our earlier studies [14].

Measurement of tissue nitrotyrosine

Nitrotyrosine abundance in the plasma was determined by Western blot analysis as described in our earlier studies [15]. The anti-nitrotyrosine antibody employed in these measurements was purchased from Upstate Biotechnology Inc (Lake Placid, New York, NY, USA). In an attempt to exclude nonspecific reactivity, the Western blot procedures were repeated with the omission of the respective primary antibodies. No reactivity was observed with the secondary antibody when primary antibody was omitted.

Measurement of xanthine oxidase activity

Xanthine oxidase activity of the liver and kidney tissues were determined as described in our earlier studies [16]. Briefly, 600 mg of liver and kidney tissues were each homogenized (30-s pulse) in 10 mL of a cold buffer solution with a Brinkmann Polytron tissue/cell disrupter (Model PT 10/35; Brinkman Instruments Inc., Westbury, NY, USA). The buffer solution contained 300 mmol/L mannitol, 20 mmol/L *N*-hydroxyethylpiperazine-*N*^{1}–2-ethanesulfonic acid (HEPES), and 13.3 mmol/L Tris (pH 7.4). Before storage at −80°C, aliquots of these hemogenates were appropriately diluted for both enzyme activity measurements and protein determination by use of the Bradford method (Bio-Rad Protein Kit).

Xanthine oxidase activity was determined in aliquots of tissue homogenates by measuring an increase in absorbance at 292 nm due to urate formed from excess xanthine used as a substrate in a pyrophosphate buffer (0.1 mol/L, pH 8). A unit of xanthine oxidase is defined as the amount of enzyme required to catalyze the formation of 1 nmol of urate per minute per milligram of protein at 21°C. The subsequent conversion of urate to allantoin and the influence of this reaction on xanthine oxidase activity measurement are presumed negligible and have been ignored because in the presence of excess xanthine, the maximum initial velocity of the reaction is being measured.

Data analysis

Analysis of variance (ANOVA), the Student *t* test and regression analysis were used in statistical evaluation of the data that are presented as mean ± SEM. P values <0.05 were considered significant.

RESULTS

General data

The CRF group exhibited a significant reduction in creatinine clearance (1.29 ± 0.34 vs. 3.72 ± 0.22 mL/min, $P < 0.01$) and a marked increase in arterial pressure. Body weight obtained at the end of the observation period was significantly lower in the CRF group (340 ± 20 g) as compared with the normal control group (398 ± 18 g). Likewise, hematocrit was significantly lower in the CRF group than that found in the sham-operated controls. The CRF group exhibited a marked reduction in urinary NO_x excretion.

Markers of oxidative stress

The CRF group showed a significant increase in plasma nitrotyrosine abundance (32.5 ± 1.9 vs. 10.2 ± 1.5 relative optical densities; $P < 0.001$). This was accompanied by a significant increase in plasma malondialdehyde (1.8 ± 0.1 vs. 1.2 ± 0.1 μmol/L, $P < 0.05$). The constellation of these findings points to enhanced ROS-mediated inactivation of nitric oxide, as well as, oxidation of lipid molecules in CRF animals.

Effect of tempol

Administration of the cell-permeable SOD-mimetic drug for one week resulted in a significant amelioration of hypertension and a significant rise in urinary NO_x excretion in the tempol-treated CRF group. Tempol administration had no significant effect on either creatinine clearance (1.35 ± 0.2 mL/min) or body weight (350 ± 8 g) in the treated CRF group. Although mean hematocrit in the tempol-treated group was higher than that found in the untreated CRF group, the difference did not attain statistical significance. Data are shown in Figure 1.

Tissue SOD protein abundance

The CRF group exhibited a significant reduction in hepatic tissue abundance of both Cu Zn SOD and Mn SOD as compared with values found in the normal control group (Figs. 2 and 3). Similarly, the CRF group

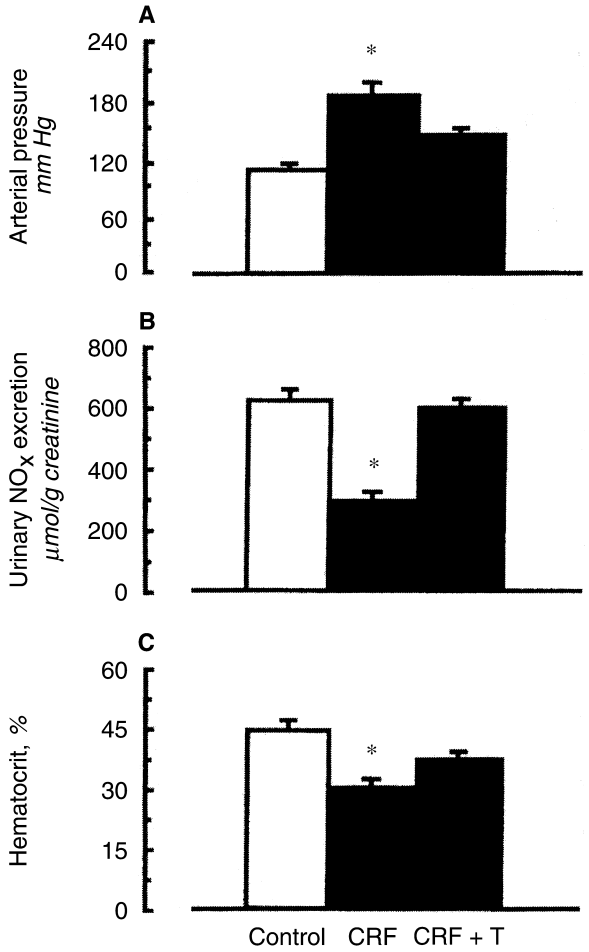

Fig. 1. Systolic arterial pressure, urinary NO metabolite (NO_x) excretion and hematocrit in sham-operated control (CTL), untreated chronic renal failure (CRF) and tempol-treated CRF (CRF+T) groups (N = 6 in each group; *$P < 0.01$).

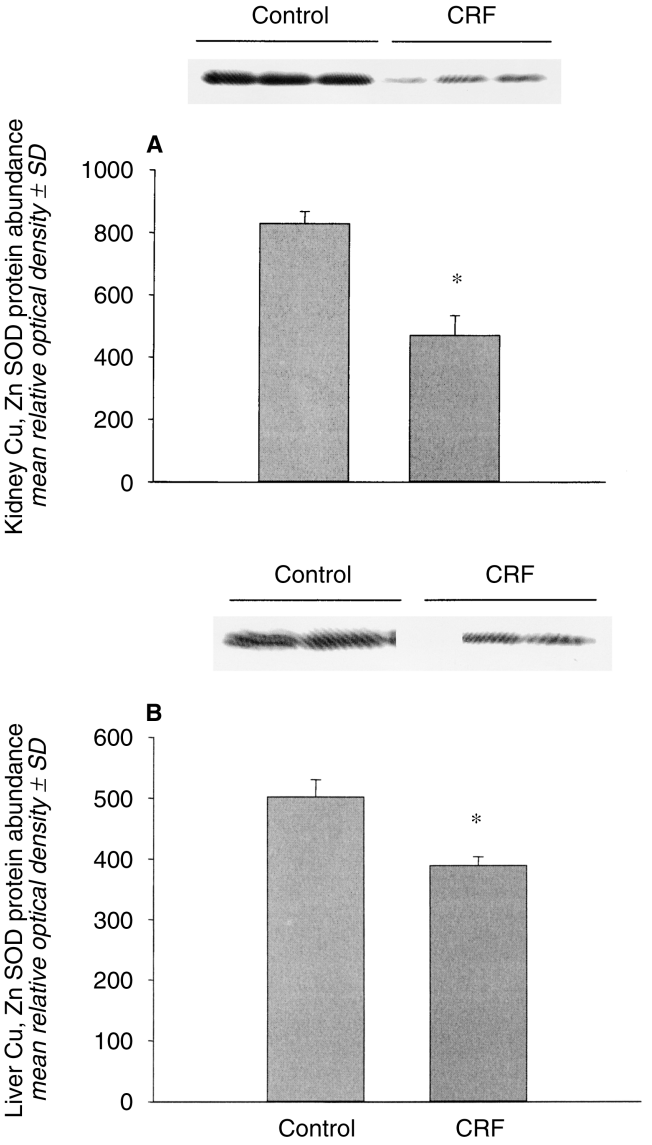

Fig. 2. Representative Western blots and group data depicting Cu Zn SOD protein abundance in the (*A*) kidney and (*B*) liver of rats with chronic renal failure (CRF) and sham-operated control (CTL) group (N = 6 in each group; *$P < 0.05$).

showed a significant reduction in the kidney tissue Cu Zn SOD and Mn SOD proteins (Figs. 2 and 3).

Tissue gp 91 phox protein and xanthine oxidase activity

The protein abundance of the gp91 phox subunit of the NAD(P)H oxidase was significantly increased in the remnant kidney and liver of the CRF animals, as compared with that found in the sham-operated controls (Fig. 4). In contrast, xanthine oxidase activity was markedly reduced in both liver and kidney tissues of the CRF animal as compared with the sham-operated controls (Fig. 5).

DISCUSSION

In confirmation of the earlier studies [2–5], the untreated CRF group showed a marked elevation of plasma malondialdehyde, which is a marker of lipid peroxidation by ROS. In addition, the untreated CRF rats exhibited a significant increase in nitrotyrosine abundance, which is the footprint of NO-ROS-protein interaction confirming our earlier studies [7]. These findings substantiated the presence of oxidative stress and ROS-mediated modification of NO, lipid and protein molecules in the CRF animals. In a series of earlier studies, we and others have shown that ROS-mediated inactivation and sequestration of NO contributes to the pathogenesis of hypertension by limiting NO availability in CRF and various other forms of genetic and acquired hypertension [5, 7, 15, 17–26]. In fact, we have recently shown that induction of oxidative stress by glutathione depletion can cause an antioxidant-remediable hypertension marked by avid

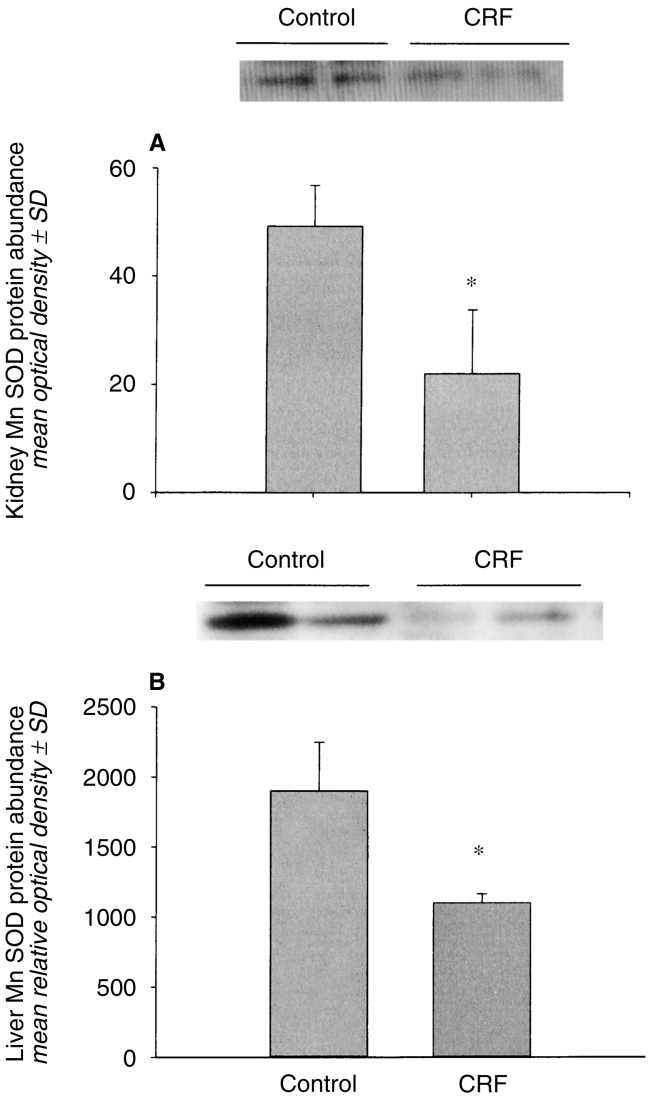

Fig. 3. Representative Western blots and group data depicting Mn SOD protein abundance in the (*A*) kidney and (*B*) liver of rats with chronic renal failure (CRF) and sham-operated control (CTL) group ($N = 6$ in each group; $^*P < 0.05$).

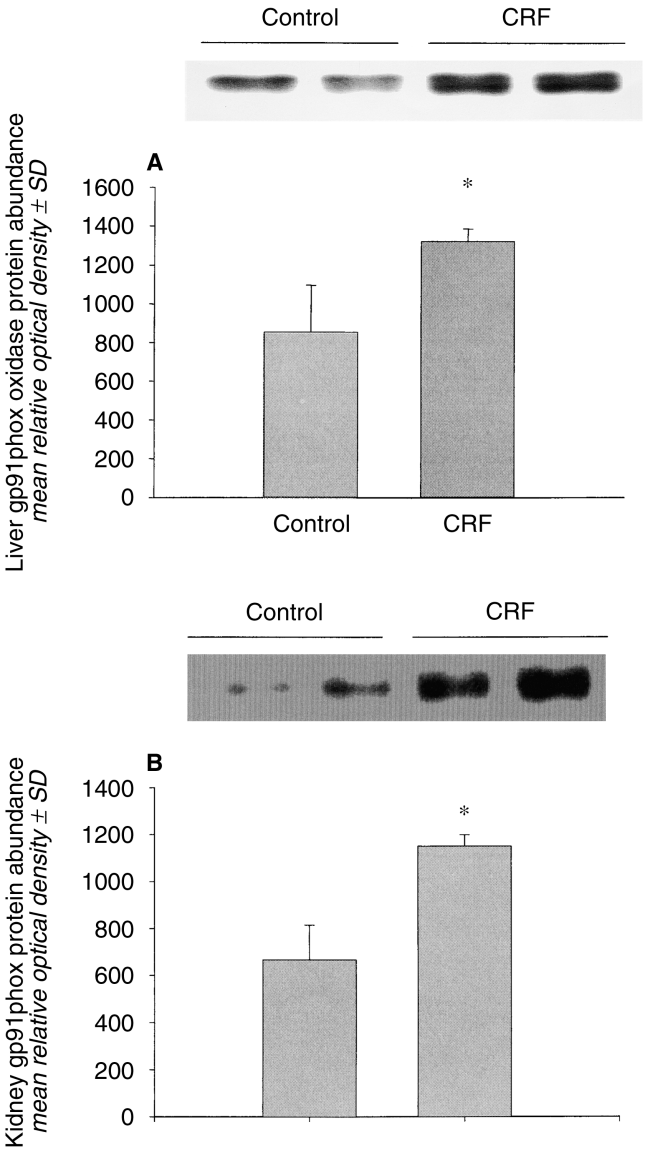

Fig. 4. Representative Western blots and group data depicting gp91 phox subunit of NADPH oxidase protein abundance in the (*B*) kidney and (*A*) liver of rats with chronic renal failure (CRF) and sham-operated control (CTL) group ($N = 6$ in each group; $^*P < 0.05$).

inactivation and sequestration of NO in genetically normal, otherwise, intact rats [25]. The latter study provided convincing evidence that oxidative stress, per se, can cause hypertension.

Administration of the cell-permeable SOD-mimetic drug, tempol, resulted in a significant fall in arterial pressure and a significant rise in urinary NO_x excretion in the CRF animals. In contrast, administration of the cell-impermeable native SOD protein carried out in our earlier studies [5] failed to significantly alter either arterial pressure or urinary NO_x excretion in this model. These observations point to the possible role of deficiency of intracellular SOD isoforms as opposed to the extracellular SOD in blood pressure regulation and NO metabolism in the CRF animals. Therefore, we determined the cytoplasmic (Cu Zn SOD) and mitochondrial (Mn SOD) isoforms in the liver and kidney of the study animals. The kidney and liver were chosen since these organs are among the metabolically most active tissues and, as such, abundantly express and heavily depend on various antioxidant enzymes especially SOD.

The CRF group exhibited a significant reduction of both Cu Zn SOD and Mn SOD protein abundance in the liver and kidney tissues. The reduction in the liver and kidney Cu Zn SOD protein abundance in the CRF animals, shown for the first time here, parallels the previously reported reduction of SOD enzymatic activity in the erythrocytes of patients with chronic renal failure

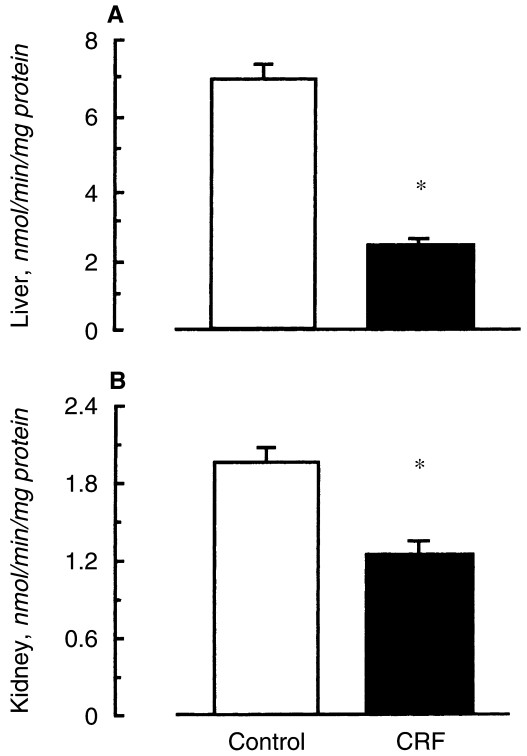

Fig. 5. Xanthine oxidase enzymatic activity of the (*A*) liver and (*B*) kidney tissues in the rats with chronic renal failure (CRF) and sham-operated control (CTL) group ($N = 6$ in each group; $^*P < 0.01$).

[9–12]. Since mature circulating erythrocytes lack nucleus and mitochondria, the reported measurements of erythrocyte SOD enzymatic activity necessarily reflect that of cytoplasmic, that is, Cu Zn SOD. Accordingly, CRF appears to cause diffuse down-regulation of Cu Zn SOD. In addition to Cu Zn SOD deficiency, the CRF group exhibited significant down-regulation of mitochondrial Mn SOD protein abundance in the kidney and liver tissues. Taken together, the data indicate that CRF results in a severe reduction of cellular SOD isoforms. Given the pivotal contribution of SOD to the cellular antioxidant defense system, the CRF-induced SOD deficiencies shown in our current study must contribute, in part, to the pathogenesis of the associated oxidative stress in this model.

The reduction in cellular SOD isoforms in the CRF animals was accompanied by a significant elevation of gp91 phox subunit of NAD(P)H oxidase, which is the major source of superoxide production in the cardiovascular tissues. Virtually all cardiovascular cell types produce ROS that play an important role in numerous physiological processes as well as in the pathogenesis of cardiovascular disease [13]. Earlier studies suggested xanthine oxidase as a source of superoxide production in the vascular tissue [27, 28]. However, recent investigations have clearly demonstrated that NAD(P)H oxidases are the main source of superoxide in cardiovascular tissues [13, 29–32]. NAD(P)H oxidases are membrane-associated enzymes consisting of two membrane-spanning subunits, gp91 phox and p22 phox, which serve as electron transfer components of the enzyme, as well as, three cytoplasmic subunits, that is, p47 phox, p67 phox and the G protein rac, which modulate the enzyme activity. These enzymes catalyze the one-electron reduction of molecular oxygen using NAD(P)H as the electron donor: $NAD(P)H + 2O_2 \rightarrow H^+ + NAD(P)^+ + 2O_2^{\bullet}$. In leukocytes, fibroblasts and endothelial cells, electrons derived from intracellular NAD(P)H is transferred to extracellular oxygen leading to production of superoxide in the extracellular space. However, in the vascular smooth muscle, superoxide is produced in the intracellular space [29, 33–35].

Increased NAD(P)H oxidase-derived superoxide production has been implicated in the pathogenesis of atherosclerotic plaque formation and rupture [13]. In addition, increased superoxide activity has been shown to play an important part in the pathogenesis of different genetic and acquired forms of hypertension in experimental animals, such as, spontaneously hypertensive rats [17, 18, 36, 37], angiotensin-infused rats [32, 38, 39], salt-sensitive Dahl rats [19] and lead-exposed rats [21]. The combination of increased NAD(P)H oxidase abundance and SOD deficiency can lead to a marked increase in superoxide activity in the CRF animals. This supposition is supported by the favorable response to administration of superoxide scavenger, tempol, in the treated CRF group.

In contrast to NAD(P)H, oxidase, xanthine oxidase activity was markedly reduced in both kidney and liver of CRF animals, confirming our earlier studies [16]. This observation argues against the role of xanthine oxide as a major source of ROS in CRF animals. As reviewed by Griendling et al [13], similar conclusions have been reached by investigators examining other models [27, 28].

The CRF animals employed in the present study exhibited a marked increase in plasma nitrotyrosine abundance, confirming our earlier studies [7]. Superoxide and other ROS avidly react with and inactivate NO, a process that can lead to functional NO deficiency and formation of highly reactive and cytotoxic products, such as, peroxynitrite and peroxynitrous acid ($O_2^{\bullet} + NO \rightarrow ONOO$) [40]. These compounds can, in turn, react with and modify various functional and structural molecules producing tissue injury and dysfunction. Therefore, alleviation of oxidative stress by antioxidant therapy should reduce ROS-mediated NO inactivation, enhance NO availability and improve hypertension. This was clearly exemplified by administration of tempol, which ameliorated hypertension and improved NO availability as evidenced by increased urinary NO_x excretion in the CRF animals employed in the present study.

In conclusion, CRF induced by renal mass reduction in genetically normal rats resulted in oxidative stress as

evidenced by accumulation of byproducts of ROS interaction with lipids (MDA), NO and proteins (nitrotyrosine). This was associated with and, at least, in part due to down-regulation of the cellular SOD isoforms (Cu Zn SOD and Mn SOD) and up-regulation of NAD(P)H oxidase, which together can raise superoxide abundance. The latter was validated by the favorable response to administration of SOD-mimetic drug, tempol, in the CRF animals.

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